# THE VAGINAL CLOSURE MEMBRANE, COPULATION, AND THE VAGINAL PLUG IN THE GUINEA-PIG, WITH FURTHER CONSIDERATIONS OF THE ŒSTROUS RHYTHM.

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Two years ago we recorded the results of a detailed study of the œstrous cycle in the guinea-pig. A rather full description of the histological and physiological changes which take place in the ovary, uterus and vagina during the "heat period" was presented. We emphasized particularly the importance of changes occurring in the microscopic composition of the vaginal fluid as indicative of the exact conditions in the uterine wall and ovarian follicles at corresponding moments.

Since that time we have somewhat extended the analysis of these phenomena. It has been found that a membrane covering the orifice of the vagina furnishes a most valuable and simple means of diagnosing certain periods in the œstrous cycle. This we have termed the "vaginal closure membrane." The exact moment of copulation and the conditions in the walls of the vagina and uterus at this time have been carefully followed, along with a consideration of the formation and significance of the vaginal plug. In the present paper a discussion of these several topics will be undertaken.

Certain points in the literature will also be discussed, a more complete review having been given in the previous article.

## 1. THE VAGINAL CLOSURE MEMBRANE.

In the former communication attention was called to the fact that "the external vaginal orifice, which during the period of œstrous activity is more or less open, actually showing in many cases a little fluid or some blood, closes and becomes less accessible after the period." During ovulation the vagina is open, but the fact of its being open is not unmistakable proof of the time of

ovulation unless the open vagina also contains what was described as second or early third stage œstrous fluid.

At that time the method of closure of the vagina following the œstrus was not explained nor was its actual significance fully appreciated. The vagina is now found to be closed by a remarkable cellular membrane and in a very definite way.

The external orifice of the vagina is crescentic in shape and the urethral opening lies in front of it in the mid line. The anterior and posterior lips of the crescent-shaped opening come together, and a delicate epithelial membrane grows over the opening and unites the lips. This occurs shortly after the heat period in females that have not copulated and in those that have copulated the closure follows the expulsion of the vaginal plug, a process to be considered beyond. The closure begins at the tips of the crescent-shaped opening and progresses toward the midpoint. The lips do not approximate so intimately at the midpoint and the membrane here seems to be under more tension than at other parts, even after the entire orifice has closed. The opening of the orifice by a tearing of the epithelial membrane begins at the strained middle part and extends from there laterally until finally the vaginal lips are freely separated. The midpoint is, therefore, the last to close, and the first to open as a general rule, although at times the opening may begin at either side of the midline.

The epithelium completely unites the lips of the vagina so that nothing can escape from or enter into the vaginal lumen without tearing this closure membrane. Such a membranous closure of the vaginal orifice is unknown to us in any other mammal. In many species the sides of the vaginal opening may be approximated or cemented together by some hardened fluid or secretion so that the lips are not readily pressed apart, but a membranous growth closing the orifice after each heat period is apparently unique.

This membrane also completely closes the vaginal opening throughout pregnancy and only becomes ruptured when the vulva swells shortly before parturition.

Such an obstruction or closure of the vaginal lumen at once suggests the hymen of the human vagina. But this, of course,

is quite a different structure in its origin as well as in its later history. The closure membrane in the guinea-pig not only exists in the young immature animal but is regularly destroyed before and reformed after every heat period that takes place during the life of the female. The formation or growth of this membrane might also be compared in some respects to the membranous growths tending to extend across and close the pharynx and other canals under pathological conditions.

The membrane is thin and delivate in structure and when stretched by slightly pressing apart the lips of the vagina with the fingers it is seen to be almost transparent, the outline of the vaginal lumen showing through. The closure membrane is of the same glossy appearance as is the surface epithelium covering the region of the vaginal lips with which it is continuous. It is composed simply of stratified squamous epithelium which has grown from the borders of the lips over the orifice and contains no vessels or blood.

When the membrane is torn or broken by accident during the diœstrum, or period of sexual rest, it reforms sometimes within a day, or within a few days, and remains until the beginning of the new period of heat or œstrus. A recognition of this membrane is then a great convenience in determining the onset of the œstrus in a group of female guinea-pigs. Daily smears of the vaginal fluid are not now necessary to find when the œstrus is about to begin in animals examined for the first time and whose rhythm is therefore unknown.

Although the presence of the closure membrane is a definite aid in recognizing the condition of the œstrous cycle, it must be remembered that this membrane often persists up to the first stage of œstrus, at which time the lumen of the vagina is filled with a mucous fluid and first stage cells. This is actually the "heat" time and the normal moment for copulation as we shall explain below. When the closure membrane still persists until the vaginal lumen is so filled it may be distended and rounded out resembling a blister membrane on the point of bursting. Puncturing this the vaginal fluid oozes out through the break. As a general rule, however, the vulva becomes inflamed and very slightly swollen immediately before œstrus and the stretched

membrane breaks. Thus the membrane has reached the breaking point or has actually broken at just about the time the female is in heat and ready to copulate.

While the presence of this membrane is a reliable index of the œstrous condition, the open vagina, or its absence, is by no means indicative of the œstrous state. Although the vagina is always open during what we have termed the second and third stages of œstrous, and, therefore, at the time of ovulation whether copulation has taken place during the first stage or not, it is nevertheless frequently open at other times. It is not permissible to assume that the open vagina indicates a state of heat or the time of ovulation in a guinea-pig. Only when the open vagina contains fluid showing on examination the cells described as second or early third stage is the ovary almost exactly in the condition of ovulation. It may be stated parenthetically that after long experience one is able as a rule to diagnose the stages of the vaginal fluid by slight difference in color and consistency without microscopic examination.

Finally, then, when the vagina is open one may only be certain of the uterine and ovarian conditions by examining the contents of its lumen, but, on the other hand, if it be closed by this membrane one may be certain that the time of the new ovulation has not yet arrived.

### 2. The Time and Manner of Copulation and the Conditions in the Reproductive Organs of the Female at this Moment.

It is well known that female guinea-pigs in common with other animals of their class, and in fact most mammals, have a definite limited time during which they accept the male, the so-called "period of heat." This period, very slightly revealed by external signs at the mouth of the vagina, but chiefly by the act of copulation has been the starting point in all previous studies on the reproductive activities of the guinea-pig. In order to prevent the modifying conditions of pregnancy following copulation, various operations have been resorted to, as in the case of some of Loeb's experiments. Such operations might complicate or even vitiate the results which follow.

In the present account we wish to describe the exact moment at which copulation takes place during the sexual cycle and to show definitely the conditions of the vagina and uterus at this moment. From the condition of the vagina or uterus the ovarian condition is readily estimated, as we have shown in the former paper. After determining the exact œstrous condition of a female at the moment she is ready for copulation we may then recognize a corresponding moment in any female by an examination of the vagina without the necessity of introducing the male or permitting copulation to occur.

In order to designate the copulation time exactly, we must review briefly the characteristics of the four very clearly defined stages of the œstrus or "heat period" proper. During stage one the uterine epithelium swells, the cells becoming distended with an abundant mucous secretion which very soon pours into the lumen and reaches the vagina. At this time a desquamation of the epithelial cells from the lower part of the vagina also begins. The second stage shows a great accumulation of leucocytes below the uterine and vaginal epithelium with a slowly progressing desquamation of epithelial cells. The third is the stage of exodus of the leucocytes, myriads of them coming through the epithelial lining of the walls of the uterus and vagina, with an accompanying extensive destruction of the epithelium. During the fourth stage the broken down epithelium falls away in masses and at the same time a regeneration of epithelium takes place beginning from the mucosa of the uterine glands.

The Graafian follicles of the ovary rupture and ovulation occurs at the end of the second stage or the beginning of the third stage, while during the fourth stage the recently ruptured Graafian follicles are already well under way in their development into new corpora lutea.

A recent abstract by Long ('19) seems to indicate that four similar stages may be recognized during the œstrus in the rat, and that these stages agree almost exactly in significance with the comparable ones in the guinea-pig: Ovulation also occurring in the rat at the end of the second or beginning of the third stage.

During our initial investigation we made no attempt to locate the exact moment of copulation and, of course, did not describe

the corresponding vaginal or uterine stage. The description by Loeb ('14), however, of the great leucocyte migration in the wall of the uterus twelve to twenty-four hours after copulation would indicate that the true "heat" or copulation occurs before the beginning of the destructive changes in the uterus, and this we now find to be true. Here and in the following section we wish to point out just how the uterine changes seem to be associated with the act of copulation, the retention of the sperm in order to insure the fertilization of the eggs, and after this the means of ridding the vagina of the excessive seminal accumulation.

A number of females have been placed with males while in one or another of the above mentioned four stages, as well as during different times of the diœstrum, or interval of sexual rest. The results show that a copulation is never accomplished except during the first stage of œstrus about twelve hours before the second stage begins. Long ('19), also finds copulation to take place in the rat during the first stage. At this stage in the guinea-pig the vagina contains a clear, foamy, saliva-like fluid in which desquamated epithelial cells of the first type are present. Differing from all other stages and times there are now no leucocytes to be found in the vaginal fluid,—compare our former Figs. 1 and 2 with Figs. 5 and 6. Even during the resting period the vagina contains some mucus but this is very scant and filled with many leucocytes, being pus-like in appearance and consistency.

To locate even more accurately the normal time of copulation the first stage may be subdivided into two shorter periods: a preparatory interval, the early beginning of the first stage, when the vagina is almost dry and contains only a scant amount of loose cells of the first type; and, second, what may be designated the true first stage, a more advanced period when the frothy mucous secretion has already begun to accumulate. Copulation takes place during this second phase of stage one and never during the first.

During stage one the vagina is characterized by two important conditions, both of which contribute to the success of copulation. In the first place the existence of a mucous secretion evidently

facilitates the act of copulation. This abundant frothy mucous accumulation is limited to the first stage, particularly to the time when copulation takes place, and it never occurs in the vagina at other times. The second contributory condition is the complete absence of leucocytes in the mucous fluid. The leucocytes begin their migration through the epithelium of the uterus and vagina at the end of the second stage. They are extremely abundant in the lumen during the third stage, while from this time on they are found in the uterus and vagina in smaller or larger quantities up to the approach of the next first stage. Two days before the first stage begins leucocytes are still plentiful, but from this time first stage epithelial cells gradually become more abundant and the leucocytes decrease in number until finally when the first stage has actually arrived no leucocyte exists in the vaginal fluid. The mucous content of the vagina during the first stage hence lacks the pus-like appearance of the vaginal fluid of the "intermenstrual" time and is clear and foamy.

The absence of leucocytes from the vaginal lumen at the time of copulation is important, since, if present, they might by their dissolving powers or phagocytic action exert an injurious effect on the spermatozoa and thus interfere with their normal function. Later a special purpose of the leucocytes seems to be to destroy the excess of spermatozoa remaining in the uterus. This frequently occurs by an interesting process of phagocytosis. A leucocyte comes in contact with a spermatozoön which with its tail is longer than the leucocyte. The leucocyte by stretching and contracting finally takes into itself the entire spermatozoön, the tail being wound in circular fashion within the cell body. The leucocytes, however, apparently accomplish most destruction by their dissolving or disintegrating action.

It seems that the migration of the leucocytes through the walls of the uterus and vagina, though not increased in extent, is accelerated by the act of copulation and the entire œstrous process is shorter than in non-copulated females. About six hours after copulation the third stage is in full development, while under virgin conditions a comparable stage is reached only after at least twelve hours from the time when copulation might have occurred. It may be said that copulation tends to

hasten ovulation, or that the act itself may facilitate the bursting of the Graafian follicles, which is a very old conception.

The act of copulation is short, lasting a few seconds only, while the preceding time of sexual excitement leading up to it is rather long. The male becomes excited by the presence of the female some time before she reaches the proper condition for copulation. A male after long isolation from females becomes sexually excited by the presence of any female irrespective or her sexual condition, and he invariably attempts to copulate. Nevertheless, the excitement of the male is not so strong nor prolonged when in the company of a female during sexual inactivity as with one during her sexual season. When the female is nearing œstrus the male is extremely excited and tries again and again to copulate, while at other times he soon tires and loses interest and ceases his aggressive behavior.

The male and female never fight during the long period of aggressiveness on the part of the male, which often lasts for many hours. The male tries to induce the female to copulate by irritation and excitement rather than by forcing her. The female may at times become nervous and attempt to bite the male, but an actual fight such as occasionally occurs between two males never takes place. No mating by force is observed; the consent of the female is necessary for the completion of copulation. Copulation is followed by a state of relaxation similar to that observed among mammals in general, and immediately afterwards both male and female may spend some time in cleaning their external genitalia.

### 3. The Vaginal Plug, Its Formation, Length of Existence and Manner of Discharge.

The spermatic fluid of the guinea-pig, especially that portion derived from the seminal vesicles, on entering the vagina of the female coagulates to form the *bouchon vaginal*, a rigid plug, filling the lumen of the vagina. This plug prevents the outflow of the sperm after every copulation. Such a vaginal plug has been described in many species of rodents and seems in general to be characteristic of this class of mammals. It was first observed in the guinea-pig by Leuckart in 1847. He correctly

described it as a Pfropf (plug), formed by the coagulation of the secretion from the seminal vesicles and serving to fill the vagina and prevent the flowing out of the sperm after copulation.

Bischoff, in 1852, verified the observations of Leuckart and accepted his conclusions regarding the rôle of the vaginal plug in the copulation process. Reichert, 1861, differed with these two original descriptions in failing to find the formation of a vaginal plug after every copulation, and concluded that its presence was not a general phenomenon. Later, however, Hensen in 1876 brought new evidence confirming the observations of Leuckart and Bischoff.

Landwehr, in 1880, examined the seminal vesicles and found their secretion to contain twenty-seven per cent. of fibrinogen to which its coagulation reaction is due. Coagulation may occur as soon as the secretion of the seminal vesicles comes in contact with a small amount of blood.

Héron-Royer, 1881, observed the vaginal plug in *Pachyuromys duprasi*, but gave no satisfactory explanation of its formation. According to him the vaginal plug was formed in the vagina before copulation and was pulled out or loosened by the hooks on the penis during the act of copulation. These observations were entirely contrary to all earlier records, according to which the plug is formed after copulation and falls out some hours later. Blanchard made histological examinations of the vaginal plugs collected by Héron-Royer and found them to consist of two parts, a central, *partie centrale*, composed chiefly of great numbers of spermatozoa, and a peripheral part, *couche corticale*, formed of hardened mucus.

Lataste, in 1882, after examinations of the vaginal plug in the same species, *Pachyuromys duprasi*, came to quite different conclusions. He states that the vaginal plug, *bouchon vaginal*, as he termed it, is not formed as Héron-Royer claimed, before copulation, but immediately after, and in the same way as was known for other rodents. Regarding its function he accepted the old opinion of Leuckart that it serves to prevent the spermatozoa from flowing out of the vagina after copulation. He also mentions an instance in which a vaginal plug-like formation was found when there had been no previous copulation. From our

observation on the œstrous discharge in the guinea-pig it is probable that this plug-like structure was nothing else than a concentrated accumulation of such a discharge, it having become unusually dense or dried out. In fact, as will be shown beyond, the superficial portion of the vaginal plug is actually the sluffed-off vaginal epithelium surrounding the coagulated seminal fluid. Thus the plug is partly of vaginal origin.

In later papers Lataste makes many contributions to the knowledge of the vaginal plug. In 1883 he described the vaginal plug in other rodents and pointed out that this formation was evidently not limited to a few species but was characteristic of the entire class.

Regarding the function of the vaginal plug, he slightly modifies his former position and concludes that its rôle is not only to prevent the sperm from flowing out of the vagina but rather by a filling up to push the sperm into the uterus. He extended the observation of Blanchard that the vaginal plug consists of two parts, differing in structure, a central core and a superficial envelope. He described the central part as consisting chiefly of the coagulated secretion of the seminal vesicles and also of a quantity of mucus, 1888*a*, while the superficial portion, *enveloppe vaginale*, was formed of stratified epithelial cells. The *enveloppe vaginale* is produced in the female by a rapid exfoliation of cells from the uterine glands and the vaginal walls on account of the irritating presence of the coagulated core. (His conception of the cause of the exfoliation is entirely incorrect.) The envelopment of the core by loosened epithelium from the vaginal wall serves to make easy the expulsion of the vaginal plug. This epithelial production he thinks is probably of a pathological nature and may be compared to the condition in women known as *vaginite exfoliante*.

These studies of more than thirty years ago by Lataste are in most respects surprisingly correct and it is only the nature of the process by which the outer epithelial envelope is formed with which we would materially differ.

Tafani, in 1888, described the vaginal plug in the mouse and found it to fall out about thirty hours after copulation.

Steinach ('94) found that the removal of the seminal vesicles

from rats did not influence their sexual instincts or ability to copulate, but decidedly impaired the power of the male to fertilize the female.

Sobotta ('95) also has studied the formation of the vaginal plug in the mouse and found it present after every copulation. Histologically it consists of an homogeneous mass which is surrounded by an envelope of vaginal epithelium. Spermatozoa are more abundant in the central mass at its upper end or that portion near the uterus as the plug lies in the vagina. He confirms the observations of Tafani regarding the fate of the vaginal plug, finding that its surface gradually becomes soft and loose and the entire mass falls out of the vagina about twenty to thirty hours after copulation. Sobotta states that the vaginal plug in the guinea-pig falls out much sooner than in the mouse, being eliminated from the vagina within from four to nine and a half hours after copulation. The longer interval is approximately correct. He claims to have at times observed another copulation following the expulsion of the first vaginal plug.

Camus and Gley ('96) studied the coagulation process in the formation of the vaginal plug. They claim coagulation to be due to the influence of a prostatic enzyme, "vesiculase," upon the secretion of the seminal vesicles. The action of the prostatic enzyme is specific towards the seminal vesicle secretion of any rodent. The prostatic enzyme of a rat will coagulate the seminal fluid of a guinea-pig and vice versa.

Rubaschkin ('05) returns to the old opinion of Reichert, 1861, in claiming that the vaginal plug is not a constant formation in the guinea-pig following copulation. His statements are as follows: Bei der Maus (Sobotta), und nach Bischoff und Hensen auch bei Meerschweinchen bildet sich nach dem Coitus ein charakteristischer Vaginal-pfropf, der auf einen vorausgegangenen Coitus hinweist. Ich muss hier die Beobachtungen von Reichert bestätigen, dass beim Meerschweinschen ein solcher Vaginalpfropf sich meistens nich bildet. Von aussen konnte ich einen klaren Pfropf in der Vagina niemals erkennen; in einigen Fällen liessen sich einige Schleimstreifen bemerken, die aber ganz unregelmässig und nicht immer zu Tage traten. In seltenen Fällen wurden nach dem Secieren Vaginalpfröpfe gefunden,

welche zum Teil aus verdichtetem Schleim, zum Teil aus Epithelzellen bestanden. Unter diesen Verhältnissen ist die Bedeutung des Vaginalpfropfs beim Meerschweinchen ganz nichtig, und am Anfange meiner Arbeit habe ich, durch diese Angabe Bischoff's irregeführt, einige Tiere verloren, weil sie zu spät getötet wurden.

Königstein ('07) described the vaginal plug in rats and agrees with the observations of Lataste, Tafani and Sobotta. He finds also the vaginal plug to consist of two parts, a central and a superficial. The vaginal plug contains in addition to the secretions of the male genital glands, mucus, detritus, many leucocytes, squamous epithelial cells in large numbers and a granular eosinphil staining secretion.

From this review the knowledge of the formation of the vaginal plug is found to be rather complete, although disagreements as to facts are expressed by several authors. It seems well established that the formation of a vaginal plug following copulation is a general phenomenon among the various species of rodents. The plug proper consists of a central core formed mainly by coagulated fluid from the seminal vesicles and this is surrounded or enclosed by a mass of flat epithelial cells, apparently derived from the vaginal wall. The coagulation of the seminal fluid may be due to the action of a prostatic enzyme although it is claimed that the coagulation occurs without the presence of such an enzyme. The vaginal plug as a whole falls out of the vagina a few hours after its formation.

On the other hand it is not clear from the literature just how or why the peripheral part of the vaginal plug, *enveloppe vaginale*, of Lataste is formed. And the manner and cause of the separation of the epithelial lining from the wall of the vagina are also unknown. These points could not be clearly understood without a knowledge of the changes occurring in the wall of the vagina and uterus during the œstrus, at which time copulation and the formation of the vaginal plug take place.

As we pointed out in our description of the œstrous changes, there is a stage in the cycle when immense numbers of leucocytes accumulate immediately below the epithelium lining the uterus and the vagina. From this position the leucocytes attack the epithelial cells and at the same time dissolve or destroy the

connection between the mucosa and the subjacent connective tissue over extensive areas. This reaction is taking place a few hours after copulation during the latter part of stage one and throughout stage two of our description. A few hours later, during stage three, the leucocytes have made still further progress in their invasion of the mucosa and the destruction of its connection with underlying tissues. In certain sections of the uterus the entire mucosa filled with immense numbers of leucocytes is completely separated from the uterine wall and lies within the lumen, while in other regions the epithelium is loosely connected but still hanging to the wall. This disconnected and degenerating mucosa loaded with leucocytes breaks into small fragments during the fourth stage and is expelled from the uterus and vagina, while a new mucosa begins to regenerate from the mouths and the regions about the uterine glands and from the deeper layers of the vaginal epithelium. This is the fate of the mucosa when no copulation has taken place.

There is, then, no pathological "vaginite exfoliante" due to an irritation of the vaginal wall by the seminal fluid as Lataste thought. But a simple periodic œstrous breaking down of the uterine wall under leucocyte invasion, entirely independent of whether copulation takes place or not.

When copulation has occurred the loss of the epithelium follows a somewhat different course. Immediately after copulation the coagulated seminal fluid forms a mass within the lumen of the vagina and partly extending into the uterus. Around this mass the mucosa forms a close fitting envelope, thus preventing its early dislocation. The envelope serves to retain the plug in the vagina until the fourth stage of the œstrous cycle at which time the enveloping epithelium becomes completely separated from the vaginal wall by the dissolving effects of the leucocytes. The epithelium is now expelled as one continuous tube forming the cover around the vaginal plug instead of sluffing off in smaller pieces as occurs during the fourth stage when a copulation has not occurred. However, the vaginal epithelium may occasionally be shed en masse without copulation. In one striking case the epithelium was pulled out of the vagina as a conical sheath, enclosing the speculum that had been introduced for examination.

It is clear, therefore, that what was termed by Lataste the "enveloppe vaginale" is the layer of epithelium separated from the underlying connective tissue by the dissolving action of the leucocytes which invade the walls of the uterus and vagina at this time. It is also readily understood how the plug, after its short sojourn in the vagina and cervix of the uterus, is finally separated from its adhesion or tight connection with the wall and expelled as a mass from the vagina.

A possible function or effect of the vaginal plug in addition to those before mentioned has recently been suggested by Long ('19). He states that a stimulation of the cervix of the uterus in rats, by merely inserting a glass rod during stage one of the œstrus, prolongs the next cycle, and suggests that the vaginal plug may also act in this mechanical way. We have not tested the prolongation of the cycle in guinea-pigs following copulation without conception as compared with its length in virgin animals.

### 4. The Œstrous Rhythm.

In our earlier review of literature it was pointed out that the knowledge of the actual time of ovulation in the guinea-pig was decidedly inexact. Nothing scarcely was known of the periodic recurrence of the œstrus stages in a given female. In short the moment of ovulation in the guinea-pig was not available for accurate experimental purposes and no definite criterion or method had been devised for detecting the œstrous condition. And this was true in spite of a very long list of studies pertaining to the reproductive activities of these animals.

Reichert, as long ago as 1861, had found that the Graafian follicles rupture about nine to ten hours after copulation. This, in general, approaches correctness, but in cases where copulation has not taken place, or failed to be observed, such knowledge is of little consequence. Rubasckhin ('05) had more recently claimed that the vagina was open and the vulva somewhat inflamed ten to twelve days after parturition, but this is certainly too short an interval to indicate an actual return of heat. It must be remembered that the female guinea-pig goes into "heat" and accepts the male almost immediately after the delivery of her litter. This fact makes the length of Rubaschkin's interval still more improbable.

The most valuable and extensive investigations of the reproductive activities of the guinea-pig were those made by Leo Loeb ('11, '14). But here the data were derived almost entirely from examinations of the uterus and ovaries after their removal from the body of the female. While such studies did give a means of comparing the conditions found among different individuals at different times, and made it possible to estimate approximately the length of the sexual periods, yet this estimate could not be transferred with certainty to any one living individual. We further objected to Loeb's method of study since it failed to permit an investigation of the recurring œstrous periods in a number of unoperated females. The results of such an investigation would be most important in determining the influence of any unusual or experimental conditions introduced with intent to modify the intervals between ovulations or other periods of the sexual cycle. These are just such problems as Loeb had under consideration.

The entire literature showed that any such thing as a regular œstrous flow was completely undiscovered for the guinea-pig. It became necessary, however, for our studies to have an accurate knowledge of ovulation times, and to determine this, extensive investigations of the sexual cycle in the guinea-pig were undertaken. A simple method of examining the vagina of the living animal proved to be of the greatest value. Virgin females were selected and the fluid present in the vaginæ was taken daily by means of a small nasal speculum and cotton swab. This fluid was smeared on slides, stained and studied microscopically. The method is fully described in the former paper.

It very soon became evident that the vagina generally contained little or no fluid, but that periodically a great accumulation of mucus and cells was to be found. This excessive amount of mucus and cells is to be recognized as a typical œstrous flow. The constituent elements of the fluid change in their relative abundance in a definite manner from the beginning to the cessation of the flow. Four clearly marked stages, as mentioned above, could be separated by microscopic examination of the fluid smears.

These changes in the composition of the vaginal fluid were

found to be associated with comparable changes in the structure of the epithelial walls of the uterus and vagina. And not only was this the case, but the changes in the vaginal fluid proved to be most reliable indices of definite processes taking place in the ovaries in connection with the rupture of the Graafian follicles and the expulsion of the ova. It is, therefore, evident that by an examination from time to time of this fluid, one may know the exact condition of the ripening follicles in the ovary and very nearly the exact moment of ovulation.

The œstrous cycles in a group of guinea-pigs were followed for a number of months in order to establish the normal periodicity or rhythm. The amount of variation that might exist in the length of the cycles in a given female was studied as well as the variations in cycle lengths among different individuals. An attempt was further made to discover any seasonal variations that might exist.

Only slight time variations were found in the periodic rhythm of a given female. For example, in one animal the record of six consecutive periods shows the œstrous flow to begin on the sixteenth day five times and on the fifteenth day once. In another case of seven consecutive periods the flow began on the sixteenth day six times and on the seventeenth day once. For further cases the reader is referred to the table given in our former paper.

There is only a limited variation in the length of the œstrous cycles among different individuals, ranging between fifteen and seventeen days in younger animals. In exceptional cases the period is slightly lengthened in older multiparæ, sometimes reaching eighteen days. These limits of fifteen and eighteen days for the lengths of the œstrous cycles have never been violated under normal conditions during the several hundred observations which we have now recorded. The method of examining the vagina for the closure membrane above described, and, in the case of its rupture, for the composition of the fluid contained within the lumen, renders these individual variations of no consequence in determining the exact "heat period" and time of ovulation.

Slight, if any, seasonal variations are shown by our animals. This may be due, however, to the uniformly warm temperature maintained in the breeding rooms during the winter months.

For a full description and photographs of the structural changes occurring in the œstrous fluid, the vagina, uterus and ovaries, the reader is referred to the original account.

After the publication of our results it was found that one of the last papers by Leo Loeb ('14), bearing on a related subject, had unfortunately been overlooked. We regret this, since a discussion of his methods and results would have been somewhat clearer in connection with our full consideration of the œstrus given in the previous paper than in the present connection. In earlier papers Loeb ('11) had completely failed to establish a definite length or periodicity for the sexual cycle in the guinea-pig. In the last paper, however, the length of the cycle was more nearly determined and a very thorough description was given of the microscopic changes taking place in the uterine wall during the heat period. Our independent account of the structural changes in the uterine wall fully confirms Loeb's description. But we are unable to agree exactly with the lengths of the sexual periods as estimated from his examinations of the removed uteri. In a still more recent article Loeb ('18) repeats his 1914 estimates and claims the lengths of the sexual cycles to vary between thirteen and a half and nineteen days.

In all cases Loeb's investigations had centered in a study of the sectioned uterus and ovary, thus necessitating their removal by operation or the death of the animal. Either procedure permits only one observation on a given female. No investigation of the uterus and vagina in the living animal had been made and no continuous observations on the consecutive cycles of given individuals were carried out.

As mentioned before, we recorded not only the structural changes of the uterus, but almost equally as marked changes in the wall of the vagina. And what we consider to be of still more importance from an analytical or experimental standpoint as a means of estimating the moment of ovulation, was the complete record of the changes in the microscopic composition of the vaginal fluid during the different stages of the sexual cycle. The removal and examination of this fluid is made without in any degree injuring the uterus or vagina and does not interfere with the further use of the female for ovulation and breeding

records. This knowledge of the definitely changing structure of the vaginal fluid made it possible to study the œstrous cycles in many living females and reduced the time element of ovulation in the guinea-pig to a certainty.

We considered in a somewhat different manner the connection between the uterine reaction and the secretion of the corpora lutea, though essentially we share Loeb's ideas of the functions of these bodies. It was concluded that the duties of the corpora lutea are probably about what Beard ('97) long ago argued in his monograph on "The Span of Gestation and the Cause of Birth."

The development and the degeneration of the vaginal and uterine mucosæ were found to follow very closely the development and degeneration of the corpora lutea in the ovaries. The case was stated as follows: "The breaking of the Graafian follicles occurs during the œstrus as a result of congestion which began in the theca folliculi at about the same time as the congestion of the stroma of the uterus and vagina. And finally when the regenerative growth of the uterine mucosa sets in, the ovaries then possess new corpora lutea in an active state of differentiation which have been derived from these recently ruptured follicles." The presence of the new active corpora lutea suppresses the final steps in the development of the almost mature Graafian follicles in both ovaries, whether the corpora lutea be located in only one of the ovaries or both. When the corpora lutea become less active and their degeneration has proceeded to a certain extent, another ovulation may then take place. Therefore, the functions of the corpora lutea are probably, first, by their presence and activity to inhibit ovulation or to determine its time, and, secondly, to preserve the structure of the uterine wall and prevent its degeneration.

Loeb has attacked the problems of corpora lutea function in the guinea-pig in a more direct experimental way than have other investigators. Yet while studying the effects of corpora lutea removal on the length of the ovulation period, he has been handicapped by the fact that his animals, after the initial operation, were later killed for examination and thus were no longer available for a continuation of the experiment. Only one observation was obtained from any particular female. The

effects on the lengths of the ovulation periods of the removal of corpora lutea or the application of its extracts could be investigated to great advantage on guinea-pigs in which the œstrous cycles are definitely known and followed through a number of consecutive periods. This could readily be done by the method before described. This method is also of value in locating the early stage of developing eggs and in making exact matings for studies on fertilization, etc.

Attention may be called to further slight objections that might be raised in considering Loeb's last paper. He studies the conditions in the structure of the uterine wall removed from females that had copulated shortly before, as well as, uteri from uncopulated females, and states, page three: "The sperm fluid present in the lumen of the uterus exerts a pressure on the surface epithelium and may thus contribute to the harmful influence of the leucocytes." This idea is incorrect since it may be clearly shown that the action of the leucocytes is equally as harmful in the destruction of the uterine epithelium during the œstrous period of virgin females.

In a similar connection Loeb also finds, page 11, that the number of leucocytes in the uterine mucosa is much smaller in animals that have not copulated. Again, page 16, "A few leucocytes can also be seen in the uterus of animals in which copulation had been prevented. . . . In such cases (non-copulated animals) also some degenerative changes occur in the uterine epithelium, but they are less marked than in animals which had copulated." These statements are not entirely in accord with our findings since there is no such marked discrepancy between virgin females and those that have copulated. Such conclusions are probably due to the fact that the uteri examined were not removed from the non-copulated females at the maximum moment of leucocyte migration and degeneration of the uterine wall (our "third stage"). Loeb had no exact means of knowing the comparable stages in copulated and non-copulated females.

The uterine mucosa of our virgin females may show leucocytes to be equally as abundant at comparable stages as the uteri from specimens after copulation. It must be recognized in this con-

nection that each stage in the condition of the uterine wall during the œstrous is of short duration and unless the uterus be removed at a given time, the abundance and position of the leucocytes and the condition of the uterine wall will be changed. Chance was against Loeb's removing the uteri from the non-copulated females at the moment of maximum leucocyte migration, since he had no exact means of knowing when this would occur without having first observed copulation.

Active migration and accumulation of leucocytes may be observed in the entire absence of sperm fluid. The rôle of this fluid and the modification of the shedding or sluffing off of the vaginal and uterine epithelium in its presence was fully brought out in the discussion above of the vaginal plug.

Loeb's estimation of the ovulation times and uterine changes from microscopic examination of fixed specimens does not make it possible to know within a few hours, or even days, of the exact moment of ovulation in a given living individual. He states, however, on page 31, that to determine the effects of the removal of the corpora lutea on the duration of the sexual cycle, it "was necessary to determine the length of the cycle in the normal guinea-pig." Not only is this necessary, particularly in view of the wide variations Loeb finds in the normal sexual cycle among different individuals, but it is better or even necessary, to know the actual length and variations of the sexual cycle in the given specimen experimented upon. As evidence of the correctness of the last statement, we may cite Loeb's method and results in determining the normal cycle lengths. This was done "by observing the time of heat of a guinea-pig and by examining the uterus and ovaries at known intervals" (after removal from different individuals). Such examinations were made on many specimens that had to be either killed or operated upon. The following ovulation intervals were thought to be the normal sexual cycles, page 31, "We found the length of the sexual period to be usually sixteen to eighteen or nineteen days; sometimes the new ovulation may take place as early as fifteen days after copulation. In two exceptional cases we observed the new ovulation as early as thirteen and a half to fourteen and a half days." The sexual cycle, therefore, varies in length from

thirteen and a half to nineteen days, a range of almost six days.

On this basis it is seen to be practically impossible to state within a day or so of when the next ovulation will take place in a female that has just passed the "heat period." And the "heat period" was very indefinitely known unless copulation had been observed. Thus, as a usual practice, in order to prevent pregnancy following a copulation used to prove the existence of heat, the oviducts were previously tied. Such a procedure might easily modify to some degree the sexual cycle and is inconvenient for further study of the animal.

Any significant experimental modification of the ovulation intervals could be readily detected by a simple examination of the microscopic structure of the vaginal fluids collected from a guinea-pig.

Finally, the "signs of heat" recorded from a report by Miss Lathrop, an animal breeder, are, according to our experience in examining and mating guinea-pigs, generally inaccurate and of little value for use in experimental studies.

## 5. Summary.

1. The œstrous cycle in the guinea-pig is very definitely limited in length. Ovulations follow one another every fifteen to seventeen days in younger individuals, while in old females the period is slightly lengthened, in exceptional cases to eighteen days.

2. These individual variations are readily controlled by the method of vaginal examination described in this and a former article, so that the actual moment of ovulation in any given female may be determined to within almost an hour of the rupture of the Graafian follicles.

3. During the period of sexual inactivity, the dioestrum, as well as during pregnancy, the orifice of the vagina is completely closed by an overgrowth of epithelium which we have termed the "vaginal closure membrane." This membrane ruptures just before or during the first stage of the œstrus in non-pregnant females and before parturition in the pregnant. It always reforms to close the vagina shortly after the "heat period" has

passed. The presence of this closure membrane is therefore positive evidence that the time of a new ovulation has not been reached. When the membrane is ruptured and the vagina open, the ovarian condition may then be determined by examination of the fluid content of the vagina as described. A knowledge of this closure membrane greatly facilitates the examination of females in locating the beginning of the œstrus.

4. Copulation takes place in the guinea-pig during stage one of the œstrous period and ovulation occurs, as we previously showed, at the end of the second stage or the beginning of the third. Long ('19) finds exactly the same to be true for the rat.

5. At the time copulation occurs, the vagina is filled with a clear foamy mucus, and this is the only time at which no leucocytes are present in the fluid. Both the nature of the vaginal fluid at this time and the absence of leucocytes contribute to the success of copulation and fertilization.

6. A vaginal plug is formed a few minutes after copulation, during the first stage, and remains in the vagina for only a short time, being expelled during the fourth stage of the œstrus.

The core or center of the vaginal plug is composed chiefly of coagulated seminal fluid. This is enclosed within an envelope of epithelial cells, being simply the sluffed-off mucosa from the wall of the uterus and vagina. This epithelial cover which is thrown off at every œstrous period is loosened or dissolved away from the uterine and vaginal wall by an enormous invasion of leucocytes which progresses from the first to the third stage. The breaking away from the vaginal wall of this enveloping epithelium causes the plug to fall out of the vagina during the fourth stage. After the expulsion of the vaginal plug, the mouth of the vagina is closed by the growth of the vaginal closure membrane.

## 6. Literature Cited.

**Beard, J.**

'97 The Span of Gestation and the Cause of Birth. A Study of the Critical Period and its Effects upon Mammals. Fischer, Jena.

**Bischoff, Th. L. W.**

'52 Entwickelungsgeschichte des Meerschweinchens. Giessen.

**Camus, L., and Gley, E.**

'96 Action coagulante du liquide prostatique sur le contenu des vésicules séminales. Soc. de Biologie, Juillet, pp. 787–788.

**Heap, W.**

'00 The Sexual Season. Quart. Jour. Mic. Soc., Vol. 44.

**Hensen, V.**

'76 Beobachtungen über die Befruchtung und Entwickelung des Kaninchens und Meerschweinchens. Zeit. f. Anat. u. Entwick., Vol. 1.

**Héron-Royer**

'81 Concrétions vagino-utérines observées chez le *Pachyuromys duprasi*. Zool. Anz., Vol. 4.

'82 A propos des bouchons vagino-utérines des Rougeurs. Zool. Anz., Vol. 5.

**Königstein, H.**

'07 Die Veränderungen der Genitalschleimhaut während der Gravidität und Brunst bei einigen Nagern. Arch. f. Physiol., Vol. 119.

**Landwehr**

'89 Ueber den Eiweisskörper, fibrinogene Substanz, der *Vesicula seminalis* der Meerschweinchen. Arch. f. Physiol., Vol. 23.

**Lataste, F.**

'82 Sur le bouchon vaginal de *Pachyuromys duprasi*. Zool. Anz., Vol. 5.

'83 Sur le bouchon vaginal des Rougeurs. Zool. Anz., Vol. 6.

'88a Matière du bouchon vaginal des Rougeurs. Compt. rend. Soc. Biol., 8th Dec., pp. 817–821.

'88b Enveloppe du bouchon vaginal des Rougeurs. Compt. rend. Soc. Biol., pp. 732–733.

'88c Enveloppe vaginale et vaginite exfoliante des Rougeurs. Compt. rend. Soc. Biol., pp. 705–707.

**Leuckart, R.**

'47 Zur Morphologie und Anatomie der Geschlechtsorgane. Göttingen.

**Loeb, L.**

'11a The Cyclic Changes in the Ovary of the Guinea-pig. Jour. Morph. Vol. 22.

'11b The Cyclic Changes in the Mammallan Ovary. Proc. Am. Phil. Soc., Vol. 50.

'14 The Correlation Between the Cyclic Changes in the Uterus and Ovaries in the Guinea-pig. BIOL. BULL., Vol. 27.

'18 *Corpus Luteum* and the Periodicity in the Sexual Cycle. Science, N.S., Vol. 48.

**Long, J. A.**

'19 The Osetrous Cycle in Rats. Proc. Am. Soc. Zoöl., Anat. Record, Vol. 15.

**Reichert, C. B.**

'61 Beiträge zur Entwicklungsgeschichte des Meerschweinchens. Abh. D. Kgl. Preuss. Akad. d. Weissensch., Berlin.

**Rubaschkin, W.**

'05 Über die Reifungs- und Befruchtungsprocesse des Meerschweincheneies. Anat. Hefte, Vol. 29.

**Sobotta, J.**

'95 Die Befruchtung und Furchung der Eies der Maus. Archiv. f. Mikrosk. Anat., Vol. 45.

**Steinach, E.**

'94 Untersuchungen zur vergleichenden Physiologie der männlichen Geschlechtsorgane, insbesondere der accessorischen Geschlechtsdrüsen. Arch. f. Physiol., Vol. 56.

**Stockard, C. R., and Papanicolaou, G.**

'17 The Existence of a Typical Œstrous Cycle in the Guinea-pig—with a Study of its Histological and Physiological Changes. Am. Jour. Anat., Vol. 22.

**Tafani, A.**

'88 La fecondatione e la segmentatione Studiate nelle uova dei Topi. Acad. Med. fisic. Florence.